Synergistic Effect of Oncogenic RET and Loss of p18 on Medullary Thyroid Carcinoma Development

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Abstract

Activating mutations in the RET proto-oncogene are associated with both familial and sporadic medullary thyroid carcinoma (MTC) development; however, the genetic mechanisms underlying MTC tumorigenesis remain largely unknown. Recently, we have identified somatic inactivating mutations in the cell cycle inhibitor gene P18 in human MTC, which coincided with activating RET mutations, suggesting a role for loss of P18 in combination with oncogenic RET in the multistep process of MTC development. Therefore, we crossed transgenic mice expressing oncogenic RET (RET2B) with mice lacking p18 (and p27, another cell cycle inhibitor) and monitored MTC development. RET2B;p18+/- mice and RET2B;p18-/- mice developed MTC with a highly increased incidence compared with their corresponding single mutant littermates. In addition, expression of oncogenic RET causes an earlier age of onset and larger MTCs in p18-/-;p27-/- mice. In a subset of MTCs of RET2B;p18-/- (p27-/-) mice, p18ink4c expression was completely lost. This loss of p18ink4c expression correlated with higher proliferation rates as well as with larger MTCs, indicating that loss of p18 in combination with oncogenic RET not only increases the risk for MTC development but also enhances MTC progression. Our data strongly indicate that oncogenic RET and loss of p18 cooperate in the multistep tumorigenesis of MTC. [Cancer Res 2008;68(5):1329–37]

Introduction

Multiple endocrine neoplasia type 2 (MEN2) is an autosomal dominantly inherited cancer syndrome, which is mainly characterized by a combination of medullary thyroid carcinoma (MTC) and adrenal pheochromocytoma. MEN2 can be subdivided into MEN2A, MEN2B, and familial MTC. Familial MTC patients solely develop MTC, whereas MEN2A and MEN2B patients may develop, in addition to MTC and pheochromocytoma, other tumors like parathyroid adenomas (for MEN2A) and mucosal ganglioneuromas (for MEN2B). MTC originates from the calcitonin-producing neuroendocrine C-cells in the thyroid gland (1). MEN2 is caused by activating germ-line mutations in the RET proto-oncogene, which encodes a transmembrane receptor tyrosine kinase (2). RET mutations associated with MEN2 lead to constitutive activation of RET (3, 4). A strong genotype-phenotype correlation has been described for RET and MEN2 (5, 6). In MEN2A, the mutations affect cysteine residues in the extracellular domain of the protein (2). In MEN2B, the most common (95%) mutation results in a Met→Thr substitution at position 918 in the intracellular domain of the protein. The M918T mutation is also found as a somatic mutation in about 30% to 40% of sporadic MTC cases (2).

Previously, we generated transgenic mice expressing the human RET oncogene with the M918T mutation in the thyroid C-cells (RET2B; ref. 7). MTCs were detected in only 13% (3 of 23) of these mice at 11 to 24 months of age, whereas C-cell hyperplasia (CCH), a premalignant stage of MTC, was observed in 77% of these mice from 8 months onward. The incomplete penetrance and variable latency period for MTC development in these mice, together with the clinical observation that carriers of the same germ-line RET mutation, can develop MTC at widely varying ages, suggesting that in addition to the mutated RET gene, other tumorigenic events are required for the development of MTC.

In human MTC, both hereditary and sporadic, the most frequently detected chromosomal alteration is loss of a specific part or the entire short arm of chromosome 1, with the most common break point on 1p32 (8–10), where the candidate tumor suppressor gene CDKN2C (from here on indicated as P18) is located. Recently, we have detected several somatic inactivating mutations in P18 in a subset of human MTCs, indicating that P18 is a tumor suppressor gene involved in human MTC development.4 p18-/- mice develop CCH and MTC, the incidence of which is enhanced by additional loss of another cell cycle inhibitor (i.e., p27; ref. 11). Both cell cycle inhibitors p18ink4c, a member of the INK4 family, and p27kip1, a member of the CIP/KIP family, inhibit the formation of active cyclin-dependent kinase (CDK) complexes and therefore phosphorylation of Rb1, a major player in G1-S phase transition (12, 13). Interestingly, p27 knockout mice as well as Rb1 knockout mice also develop CCH and MTC, indicating that the CDK-Rb pathway is involved in the tumorigenesis of MTC (11, 14–16). Moreover, somatic RET mutations have been described in MTC of Rb1p53 knockout mice (17), suggesting a cooperative effect of oncogenic RET and the Rb pathway in MTC tumorigenesis.

Note: Supplementary data for this article are available at Cancer Research Online (http://cancerres.aacrjournals.org/).

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Because of the development of MTC in both RET2B transgenic mice and p18, p27 and p18p27 knockout mice, together with the simultaneous occurrence of activating RET mutations and inactivating P18 mutations in human MTCs, we hypothesized that p18 and/or p27 may collaborate with oncogenic RET in the multistep tumorigenesis of MTC. To address this hypothesis, we crossed RET2B mice with p18-/mice and monitored MTC development. Our results indicate a strong synergistic effect of oncogenic RET and loss of p18 on MTC development and progression.

Materials and Methods

Mouse strains and genotyping. All experiments with mice were done with the approval of the Animal Experimental and Ethics Committee of the University of Utrecht, Utrecht, the Netherlands. RET2B transgenic mice (C57BL/6 background) and p18p27 knockout mice (C57BL/6 background) were previously described (7, 11, 18). For genotype verification, genomic tail-DNA was isolated with Prot-K lysis buffer [100 mmol/L Tris (pH 8), 5 mmol/L EDTA, 0.2% SDS, 0.2 mol/L NaCl, 100 μg/μL proteinase K] at 55°C. Separate PCRs were done for RET, p18, and p27 using primers listed in Supplementary Table S1. All PCRs were done at an annealing temperature of 55°C for 33 cycles.

Plasma calcitonin measurements. Blood was collected every 3 months via orbital puncture after isoflurane anesthesia. Plasma was isolated from EXD2-blood by centrifugation. Plasma calcitonin concentrations were measured using a solid-phase, enzyme-labeled, two-site chemiluminescent immunometric assay (Siemens Diagnostics) with an Immulite 1000 analyzer according to the manufacturer’s protocol. Interassay variability (measured over 18 assays) shows that this assay is very sensitive and robust: average concentration for assay-control 1, 1.12 pg/mL (SD, 0.27 pg/mL); variation coefficient, 2.24%; average concentration for assay-control 2, 209.5 pg/mL (SD, 3.99 pg/mL); variation coefficient, 1.90%. Plasma calcitonin levels were measured in counts per second (cps) and calculated to picograms according to the assay standard curve. Wild-type non–MTC-bearing mice have undetectable plasma calcitonin levels (<35,000 cps). Histologic analysis revealed that in our study, plasma calcitonin levels ≥1.4 pg/mL (≥100,000 cps) indicated microscopically detectable MTCs.

Tissue processing and immunohistochemistry. Isolated thyroid tissues were fixed in 4% formaldehyde (Klinipath) overnight, dehydrated, embedded in paraffin, and sections of 6 μm. For p27kip1 and PCNA staining, antigen unmasking was done by boiling the slides in sodium citrate buffer (10 mmol/L sodium citrate trisodium salt dihydrate in distilled water, pH 6) for 15 min. Primary antibodies used were rabbit polyclonal anti-calcitonin antibody (DAKO), diluted 1:5,000; rabbit polyclonal anti-p18 antibody (M-168; Santa Cruz Biotechnology), diluted 1:50; mouse monoclonal anti-KIP1 antibody (BD Transduction Laboratories), diluted 1:500; and mouse monoclonal anti-PCNA antibody (PC10; Cell Signaling Technology), diluted 1:1,000.

Secondary antibodies used were horseradish peroxidase (HRP)–conjugated swine anti-rabbit antibody (DAKO) and HRP-conjugated rabbit anti-mouse antibody (DAKO), diluted 1:100, at room temperature for 30 min. For p27kip1 staining, goat anti-M/Ra IgG DVPDO-500H (Powervision; ImmunoLogic) was used as secondary antibody. After 3,3′-diaminobenzidine precipitation, a hematoxylin counterstaining was done. The anti-KIP1 antibody and Powervision were kindly provided by the Department of Pathology of the University Medical Center Utrecht, Utrecht, the Netherlands.

Laser capture microdissection. Laser capture microdissection was used to obtain cell populations of selected areas from paraffin-embedded tissue sections. Sections (10 μm thick) on 1-mm PEN Membrane Slides (P.A.L.M. Microlaser Technologies) were deparaffinized and lightly stained with hematoxylin. Using a P.A.L.M. Microbeam laser capture microdissection system (P.A.L.M. Microlaser Technologies), lesions of MTCs and normal tissues were separately isolated from the sections. DNA was isolated from the microdissected tissue samples with Prot-K lysis buffer [50 mmol/L Tris-HCl (pH 8), 0.5% Tween 20, 2 mg/mL Prot-K] at 55°C for 16 h.

Results

Generation of compound RET2B;p18, RET2B;p27 and RET2B;p18p27 mice. To investigate the role of oncogenic RET, p18, and p27 in the multistep process of MTC development in vivo, we crossed RET2B mice (7) with p18-/;p27-/ knockout mice (18). Mating between these mouse strains, both on a C57BL/6 background, yielded all expected genotypes at the anticipated Mendelian ratios. Reproduction was not affected in any of the generated genotypes. For this study, only mice from the third generation were used.

Wild-type mice (WT; n = 26), RET2B mice (n = 48), p18+-/ mice (n = 25), p18-/;p27-/ mice (n = 30), and p27-/ mice (n = 22) were generated. To study the effect of oncogenic RET with additional loss of either p18 or p27 on MTC development, compound RET2B;p18-/;p27-/- mice (n = 44), RET2B;p18-/-;p27+/+ mice (n = 31), and RET2B;p27-/-;p27+/- mice (n = 49) were generated. Franklin et al. (11) showed a synergistic effect of loss of both p18 and p27 on MTC development. To investigate a putative additional effect of oncogenic RET on loss of both p18 and p27, we generated p18-/-;p27+/+ mice (n = 20), p18-/-;p27-/- mice (n = 24), RET2B;p18-/-;p27+/+ mice (n = 44), and RET2B;p18-/-;p27-/- mice (n = 40). p27-/- mice were not generated because in combination with homozygous loss of p18, they have a low mean survival of 3.5 months (11,18).

p18-/;p27-/- mice with and without additional loss of p27 display gigantism from early ages onward (11,18). We did not detect any apparent enhancement of the gigantism phenotype in the presence of oncogenic RET. All mice were monitored up to 12 months of age. In this period, survival was not affected in the different genotype groups, except for p18-/-;p27-/- mice, which died, or became moribund, at the age of 8 to 9 months due to previously described causes (11,18). The survival of compound RET2B;p18-/-;p27-/- mice was comparable to the survival of p18-/;p27-/- mice. Therefore, MTC development in the latter two genotype groups was monitored up to 9 months of age.

Synergistic effect of oncogenic RET and loss of p18 on MTC development. In RET2B mice, the expression of the oncogenic RET transgene is directed to the thyroid C-cells due to the used CALC-I promoter. These mice develop CCH and MTC without any other tumors involved in MEN2 (7). Here, we monitored the
development of MTC in the different genotype groups by measuring plasma calcitonin levels of the mice. Plasma calcitonin levels are used as a specific tumor marker for the clinical diagnosis and follow-up of MTC patients (19). Previously, we showed that plasma calcitonin levels can also be used to monitor MTC development in mice (7). Blood was drawn every 3 months and when the mice became moribund. In this way, we were able to monitor MTC development and progression in time. At the time of sacrifice, thyroid glands were isolated for histologic analysis. Calcitonin immunostainings were done to identify the presence of a MTC. Bilateral and multifocal MTCs were detected in mice of different genotype groups. The level of plasma calcitonin in wild-type non–MTC-bearing mice was undetectable (Fig. 1A). Most MTCs were detectable only by microscopy (Fig. 1B and C); however, some mice developed macroscopically detectable MTCs (Fig. 1D).

The numbers and percentages of mice of all different genotype groups that developed MTC within 12 months are summarized in Table 1. Up to 12 months of age, no MTC was detected in the WT, RET2B, p18−/−, and p27−/− groups, whereas 11% (3 of 27) of p18−/− mice had developed MTC at this age. Forty-three percent (13 of 30) of compound RET2Bp18−/− mice displayed MTC at 12 months, which is a significantly increased incidence compared with both RET2B and p18−/− mice: 4% (2 of 47) of RET2Bp27−/− mice developed MTC up to 12 months of age (Table 1), indicating that heterozygous loss of p18 is not sufficient for MTC development within this period. However, compound RET2Bp18−/− mice displayed a significantly increased MTC incidence already at 9 months (21%; 9 of 42) as compared with both RET2B and p18−/− mice, suggesting that p18 is haploinsufficient in the presence of oncogenic RET (Table 1).

RET2B mice, which had lost a single p27 allele, did not show a significant increase in MTC incidence compared with RET2B mice and p27−/− mice: 4% (2 of 47) of RET2Bp27−/− mice developed MTC up to 12 months of age (Table 1), indicating that heterozygous loss of p27 does not affect RET-induced MTC development. These data suggest that RET and p18, but not RET and p27, cooperate in MTC development.

CHH in RET2Bp27−/− mice and p18−/−p27−/− mice at 12 months of age. Up to 12 months of age, WT mice did not develop MTC, nor could we histologically detect CHH in these mice. The mean plasma calcitonin level of WT mice at the age of 12 months was indicated as 34,842 cps (SD, 6,466 cps; n = 25). To investigate whether mice of the different genotype groups that did not develop MTC (plasma calcitonin <100,000 cps) developed

**Figure 1.** MTC development in mice with elevated plasma calcitonin levels. A, calcitonin (CT) staining on the thyroid gland of a wild-type C57BL/6 mouse with undetectable plasma calcitonin level indicating normal C-cell amounts. Right, higher magnification of the boxed area in left. B, calcitonin staining on a thyroid gland of a mouse with a plasma calcitonin level of 1.7 pg/mL indicating a micro-MTC. Right, higher magnification of the boxed area in left. C, calcitonin staining on thyroid glands of mice with plasma calcitonin levels of 2.6, 10.4, and 158 pg/mL, indicating MTCs of increasing sizes. D, macroscopic bilateral MTC (white arrows) in a mouse with a plasma calcitonin level of 569 pg/mL. Black arrows, calcitonin-positive C-cells. f, thyroid follicle; t, trachea. Bar, 50 μm.
CCH, we analyzed whether they had significantly elevated plasma calcitonin levels at the age of 12 months (Table 2).

Plasma calcitonin levels of RET2B mice, p18+/− mice, and p27+/− mice did not significantly differ from those of WT mice, suggesting that these mice did not develop CCH at the age of 12 months. P18−/− mice, however, did develop a significant increase in plasma calcitonin at the age of 12 months (52.271 cps; SD, 17.043 cps; n = 24), suggesting CCH. In the three RET2Bp18 genotype groups in which MTC was also detected at 12 months of age, increased plasma calcitonin levels were identified in mice that did not develop MTC.

Interestingly, in the RET2Bp27+/− group and p18−/−p27+/− group, in which MTC development could (almost) not be observed at 12 months, CCH was suggested by elevated plasma calcitonin levels compared with those of WT mice. Comparing plasma calcitonin levels of RET2Bp27−/− mice with plasma levels of RET2B mice revealed an increase that was not significant (P = 0.06), whereas plasma calcitonin levels of p18−/−p27+/− mice were significantly (P < 0.001) increased compared with those of p18+/− mice. These data suggest that loss of p27 synergizes with loss of p18, but not with oncogenic RET, in MTC development.

Oncogenic RET causes earlier MTC onset in compound transgenic mice. The synergism between p18 and p27 could also be observed in p18−/−p27+/− mice: 88% (15 of 17) of p18−/−p27+/− double knockout mice developed MTC at 9 months of age, compared with 4% (1 of 28) of p18+/− mice (Table 1), indicating that simultaneous loss of p18 (homozygous) and p27 (heterozygous) is sufficient for MTC development in mice, as was also previously shown by Franklin et al. (11). We wondered whether oncogenic RET could increase the MTC incidence even further in the p18p27 background. One hundred percent (26 of 26) of RET2Bp18−/−p27−/− mice developed MTC at 9 months, which was not significantly increased compared with p18−/−p27−/− mice at this age [88% (15 of 17); Table 1]. However, 27% (12 of 44) of RET2Bp18−/−p27−/− mice displayed MTC at 12 months of age, which is a significant increase compared with 0% (0 of 18) of p18−/−p27−/− mice (Table 1). In addition, a remarkable difference in MTC incidence between p18−/−p27−/− mice and RET2Bp18−/−p27+/− mice was observed at the age of 6 months: 31% (11 of 35) of RET2Bp18−/−p27+/− mice developed MTC at this age compared with 0% (0 of 23) of p18−/−p27+/− mice (Table 1), strongly indicating that oncogenic RET causes an earlier MTC onset in p18−/−p27+/− mice. All mice that displayed MTC already at the age of 3 or 6 months (n = 15) belonged to groups with a combined RET2B and heterozygous or homozygous p18 knockout genotype (Table 1), confirming that the combination of oncogenic RET and loss of p18 strongly promotes (early) MTC development in mice.

Oncogenic RET induces larger MTCs in compound transgenic mice. As described above, oncogenic RET in combination with loss of p18 or loss of both p18 and p27 resulted in increased

<table>
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<th>6 mo [n (%)]</th>
<th>9 mo [n (%)]</th>
<th>12 mo [n (%)]</th>
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<td>2/30 (7)</td>
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<td>11/35 (31)⁵,⁶,⁷</td>
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NOTE: MTC was diagnosed by plasma calcitonin levels >1.4 pg/mL.

Abbreviation: ND, not determined (mice were sacrificed at 9 mo).

*P = 0.001 for RET2Bp18+/− vs RET2B; P = 0.014 for RET2Bp18+/− vs p18+/−.
P < 0.001 for RET2Bp18+/− vs RET2B; P = 0.006 for RET2Bp18+/− vs p18+/−.
P < 0.001 for RET2Bp18−/+ vs RET2B; P = 0.004 for RET2Bp18−/+ vs p18−/+.
P < 0.001 for RET2Bp18+/− vs RET2B; P = 0.007 for RET2Bp18+/− vs p18−/+.
P = 0.269 for RET2Bp27+/− vs RET2B; P = 0.473 for RET2Bp27−/− vs p27−/−.
P = 0.258 for RET2Bp27+/− vs RET2B; P = 0.475 for RET2Bp27+/− vs p27−/−.
**P < 0.001 for P18+/−p27−/− vs p18+/−; P < 0.001 for p18+/−p27−/− vs p27−/−.
††P = 0.117 for RET2Bp18+/−p27−/− vs RET2B; P = 0.334 for RET2Bp18+/−p27−/− vs p18+/−p27−/−.
††P = 0.039 for RET2Bp18+/−p27−/− vs RET2Bp18−/−p27−/−.
||P < 0.001 for RET2Bp18+/−p27+/− vs RET2B; P = 0.01 for RET2Bp18+/−p27+/− vs p18+/−p27+/−.
|PP < 0.533 for RET2Bp18+/−p27−/− vs RET2Bp18−/−p27−/−.
|PP < 0.001 for RET2Bp18−/−p27−/− vs RET2B; P = 0.015 for RET2Bp18−/−p27−/− vs p18−/−p27−/−.
|†††P < 0.001 for RET2Bp18−/−p27−/− vs RET2B; P = 0.151 for RET2Bp18−/−p27−/− vs p18−/−p27−/−.
|†††P < 0.001 for RET2Bp18−/−p27−/− vs RET2Bp18−/−p27−/−.
MTC incidence as well as decreased age of onset. Next, we investigated whether we could detect an effect on MTC growth of oncogenic RET, in addition to loss of p18 and p27, by comparing plasma calcitonin levels of mice with MTC. We found a correlation between plasma calcitonin levels and MTC size, as was also detected for human MTC patients (Fig. 1C; ref. 21). In Fig. 2, plasma calcitonin levels of all mice with MTC from different compound genotype groups at 3, 6, 9, and 12 months of age are indicated.

At the age of 9 months, plasma calcitonin levels of compound RET2Bp18<sup>+/−</sup>/p27<sup>−/−</sup> mice with MTC (n = 24) were significantly higher (ranging from 1.4 to 569 pg/mL with a median concentration of 84.25 pg/mL; P < 0.01) compared with plasma calcitonin levels of p18<sup>−/−</sup>/p27<sup>−/−</sup> mice with MTC (n = 15; ranging from 2.1 to 261 pg/mL with a median concentration of 15.6 pg/mL; Fig. 2). This indicates that already at 9 months of age, RET2Bp18<sup>−/−</sup>/p27<sup>−/−</sup> mice have larger MTCs compared with p18<sup>−/−</sup>/p27<sup>−/−</sup> mice. This was also confirmed by histologic analysis.

To investigate whether we could detect a difference in growth rate in these MTCs, we analyzed proliferation in five MTCs of both p18<sup>−/−</sup>/p27<sup>−/−</sup> and RET2Bp18<sup>−/−</sup>/p27<sup>−/−</sup> mice. The MTCs were selected from mice of each genotype group, which matched according to their plasma calcitonin levels, and thus MTCs were of comparable sizes. As determined by PCNA immunostainings and subsequent proliferation index calculations, we did not identify a significant difference (P = 0.43) in the average proliferation rates between MTCs from the two different genotype groups: 17.7% (SD, 2.4%) for MTCs from p18<sup>−/−</sup>/p27<sup>−/−</sup> and 19.6% (SD, 4.6%) for MTCs from RET2Bp18<sup>−/−</sup>/p27<sup>−/−</sup> mice (Supplementary Table S2), suggesting that the larger MTCs detected in RET2Bp18<sup>−/−</sup> mice are due to earlier age of onset rather than to increased growth rate.

**Heterozygous loss of p27 increases MTC incidence in RET2B; p18<sup>−/−</sup> but not RET2Bp18<sup>−/−</sup> compound transgenic mice.** As described above, heterozygous loss of p27 additionally affects MTC development in combination with complete loss of p18, but not with oncogenic RET. At 9 months of age, RET2Bp18<sup>−/−</sup>/p27<sup>−/−</sup> mice displayed a significantly higher MTC incidence (100%; 26 of 26) compared with RET2Bp18<sup>−/−</sup> mice [33% (10 of 30); Table 1]. In addition, RET2Bp18<sup>−/−</sup>/p27<sup>−/−</sup> mice with MTC also displayed significantly higher (P < 0.001) plasma calcitonin levels compared with RET2Bp18<sup>−/−</sup> mice with MTC (n = 10; ranging from 1.7 to 420 pg/mL with a median concentration of 2.2 pg/mL), indicating an additional effect of heterozygous loss of p27 on RET2Bp18<sup>−/−</sup>–induced MTC development (Fig. 2).

Comparing plasma calcitonin levels of RET2Bp18<sup>−/−</sup>/p27<sup>−/−</sup> mice that did not develop MTC at the age of 12 months with plasma calcitonin levels of RET2Bp18<sup>−/−</sup> mice revealed an increase that was not significant (P = 0.07), suggesting that additional loss of p27 does not strongly enhance CCH in RET2Bp18<sup>−/−</sup> mice (Table 2). Surprisingly, RET2Bp18<sup>−/−</sup>/p27<sup>−/−</sup> mice (7%; 3 of 44) displayed a significantly lower MTC incidence at the age of 9 months compared with RET2Bp18<sup>−/−</sup> mice (21%; 9 of 42), suggesting a protective effect of loss of p27 on MTC development (Table 1). However, five of these nine RET2Bp18<sup>−/−</sup>/p27<sup>−/−</sup> mice displayed plasma calcitonin levels of 1.4 to 2 pg/mL, indicating very small MTCs that did not develop MTC at the age of 12 months with plasma calcitonin levels of 1.4 to 569 pg/mL with a median concentration of 84.25 pg/mL; Fig. 2). This suggests that the larger MTCs detected in RET2Bp18<sup>−/−</sup> mice are due to earlier age of onset rather than to increased growth rate.

![Table 2. Elevated plasma calcitonin levels indicating CCH in mice that did not develop MTC (plasma calcitonin <100,000 cps) at 12 mo of age](image)
from this tumor (Supplementary Fig. S1). Subsequently, we carried out a mutation analysis on DNA isolated from all nine \( p18^{+/+} \) MTCs. In none of these MTCs a mutation in the coding region of \( p18 \) could be detected.

Coxon et al. (17) detected somatic \( RET \) mutations in four of nine MTCs from \( Rb;p53 \) double knockout mice. These \( RET \) mutations corresponded with human MEN2A-associated \( RET \) mutations. To investigate whether such mutations were acquired in MTCs from \( p18; p27 \) knockout mice as well, we carried out nucleotide sequence analysis on DNA isolated from MTCs of seven \( p18^{+/+}; p27^{+/+} \) mice. In none of these MTCs a somatic \( RET \) mutation could be identified.

Loss of \( p18^{\text{ink4c}} \) expression in MTCs from \( p18^{+/+} \) mice correlates with MTC progression. To assess whether \( p18^{\text{ink4c}} \) expression was lost in MTCs of heterozygous \( p18 \) knockout mice, \( p18^{\text{ink4c}} \) expression was determined in MTCs of seven \( p18^{-/-}; p27^{-/-} \) mice. In none of these \( p18^{-/-}; p27^{-/-} \) mice, we could detect loss of the wild-type \( p18 \) allele(s) (Supplementary Fig. S1), nor could we detect mutations in the coding region of \( p18 \) in MTCs from these \( p18^{-/-}; p27^{-/-} \) mice, indicating that other mechanisms are involved in the loss of \( p18^{\text{ink4c}} \) expression in these MTCs.

Furthermore, we investigated whether loss of \( p18^{\text{ink4c}} \) expression affected MTC growth by comparing the plasma calcitonin levels of \( RET2B;p18^{+/+}; p27^{+/+} \) mice with MTCs with (partial) loss of \( p18^{\text{ink4c}} \) expression (10; ranging from 6.2 to 522 pg/mL with a median concentration of 97 pg/mL) to plasma calcitonin levels of \( RET2B;p18^{+/+}; p27^{+/+} \) mice with \( p18^{\text{ink4c}} \) expression (7; ranging from 1.7 to 19.5 pg/mL with a median concentration of 4.8 pg/mL; Table 3). The plasma calcitonin levels of mice with MTCs without \( p18^{\text{ink4c}} \) expression were significantly higher \((P = 0.001)\) compared with those of mice with MTCs expressing \( p18^{\text{ink4c}} \), indicating that MTCs without \( p18^{\text{ink4c}} \) expression were larger compared with those expressing \( p18^{\text{ink4c}} \).

Next, we investigated whether this somatic loss of \( p18^{\text{ink4c}} \) expression was due to LOH or somatic mutations in the \( p18 \) gene. In none of the MTCs that showed (partial) loss of \( p18^{\text{ink4c}} \) expression could we detect loss of the wild-type \( p18 \) allele(s) (Supplementary Fig. S1), nor could we detect mutations in the coding region of \( p18 \) in MTCs from these \( 17 RET2Bp18^{-/-}p27^{-/-} \) mice, indicating that other mechanisms are involved in the loss of \( p18^{\text{ink4c}} \) expression in these MTCs.

Next, we determined the proliferation rates of MTCs of \( RET2B;p18^{+/+}; p27^{+/+} \) mice with and without \( p18^{\text{ink4c}} \) expression.

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**Figure 2.** Plasma calcitonin levels of mice with MTC (≥1.4 pg/mL) from compound genotype groups are indicated at 3, 6, 9, and 12 mo of age. A logarithmic scale was used to plot the plasma calcitonin levels. ND, not determined (mice were sacrificed at 9 mo).
The average proliferation index of MTCs that had (partially) lost p18\textsuperscript{Ink4c} expression \([n = 10; 17.9\% \text{ (SD, 4.6)}]\) resembled the average proliferation index of MTCs of RET2B;\textsubscript{p18}\textsuperscript{+/−} (\textsubscript{p27}\textsuperscript{+/−}) mice (19.6\%; SD, 4.6\%) as shown in Supplemental Table S2. This was significantly higher \(P = 0.007\) compared with the average proliferation index of MTCs of RET2B;p18\textsuperscript{−/−} (\textsuperscript{p27}−/−) mice that express p18\textsuperscript{Ink4c} \([n = 6; 11.5\% \text{ (SD, 2.5)}];\) Table 3. In conclusion, loss of p18\textsuperscript{Ink4c} expression correlates with larger MTCs and higher proliferation rates, indicating that loss of p18\textsuperscript{Ink4c} enhances MTC progression.

**Discussion**

In this article, we provide experimental evidence that p18 inactivation functionally collaborates with oncogenic RET in murine MTC development. As compared with the single mutant mouse strains, we have found an increased MTC incidence in both RET2B;p18\textsuperscript{+/−} and RET2B;p18\textsuperscript{−/−} mice from 9 months onward. Heterozygous loss of p27 did not result in increased MTC incidence in RET2B mice up to 12 months of age. This indicates that loss of p18, but not loss of p27, cooperates with oncogenic RET in MTC development. The observed earlier age of onset of MTC, as well as the larger MTCs, in compound RET2B;p18\textsuperscript{+/−} mice provides further evidence for synergism between oncogenic RET and loss of p18. Furthermore, somatic loss of p18\textsuperscript{Ink4c} expression in MTCs of heterozygous p18 knockout mice resulted in higher proliferation rates and larger MTCs. Loss of p18 in the presence of oncogenic RET greatly enhances MTC incidence as well as MTC progression, which shows that loss of p18 is a frequent and severe additional oncogenic hit in RET-induced MTC tumorigenesis in mouse.

Interestingly, our results are consistent with a previously reported study about the role of p18 and p27 in MEN1 (23). p18, but not p27, collaborates with Men1 to suppress tumor formation in pituitary, testis, thyroid, parathyroid, and pancreatic islets (23). The synergistic effect of loss of p18 on tumor formation in both Men1 knockout mice and RET2B transgenic mice indicates a general tumor suppressor role for p18 in endocrine tissues. It has been proposed that INK4 proteins regulate the cell cycle in a cell lineage–specific manner due to tissue-specific differences in expression patterns (24). In contrast to most other INK4 proteins, p18\textsuperscript{Ink4c} is expressed during mouse embryogenesis as well as in most adult tissues like testis, spleen, kidney, skeletal muscle, and lung (25). Therefore, the tissue-specific effect of loss of p18 on endocrine tumorogenesis is not likely due to an endocrine-specific expression pattern of p18\textsuperscript{Ink4c}. More likely, a lack of redundancy involving the other INK4 proteins in endocrine tissues might explain the particular importance of p18 in these tissues.

Previously, biochemical studies have revealed that loss of menin, the Men1 gene product, down-regulates p18\textsuperscript{Ink4c} and p27\textsuperscript{Ki67} expression (26, 27). Previously, we have detected that expression of oncogenic RET results in down-regulation of p18\textsuperscript{Ink4c} and p27\textsuperscript{Ki67} expression, leading to increased proliferation (28). Therefore, the observed (partial) loss of p18\textsuperscript{Ink4c} expression in a subset of MTCs from p18\textsuperscript{−/−} mice without loss of the remaining p18 allele might be caused by oncogenic RET signaling. However, this oncogenic RET signaling did not result in loss in p27\textsuperscript{ni67} expression in MTCs from p27\textsuperscript{−/−} mice.

Several reports have shown that p18\textsuperscript{Ink4c} and p27\textsuperscript{Ki67} cooperate in cell cycle arrest of different cell types (29, 30). Loss of inhibition both early and late in G\textsubscript{1} (e.g., by loss of p18\textsuperscript{Ink4c} and p27\textsuperscript{Ki67}, respectively) affects cell cycle progression more severely compared with loss of inhibition early in G\textsubscript{1} alone (31), which could explain the differential effects observed in RET2B;p18\textsuperscript{−/−} mice and RET2B;p27\textsuperscript{−/−} mice. Different double knockout mouse models, in addition to p18p27 knockout mice, like Rbp53 knockout mice (16, 32), p18p53 knockout mice, p27p53 knockout mice (33), and Rbp27 knockout mice (14), have revealed a synergism between cell cycle regulatory genes in MTC development. This is the first time that synergism between a receptor tyrosine kinase (RET) and a CDK inhibitor (p18\textsuperscript{Ink4c}) is reported to be involved in the
multistep process of MTC development. Loss of p27 in compound
RET2B;p18−/− mice further increases MTC incidence, which is not
associated with loss of p27Kip1 expression, indicating that p27 is a
haploinsufficient tumor suppressor gene in p18- and RET2B;p18−
induced MTC development.

Involvement of oncogenic RET in human MEN2 and MTC
development is already well established. Our data suggest a
cooperative role for P18 and RET in human MTC tumorigenesis.
Germ-line P18 mutations have not yet been described in man,
and somatic alterations in the P18 gene have rarely been detected
in human cancer. However, loss of chromosome 1p32, the loca-
tion of the P18 gene, is associated with both sporadic and familial
MTC (8–10). Recently, we have detected somatic inactivating
mutations in sporadic as well as hereditary MTCs, with a
relatively high frequency compared with other types of human
cancer investigated. Interestingly, all somatic P18 mutations
detected in human MTC coincided with germ-line or somatic
RET mutations, suggesting that a cooperation between oncogenic
RET and inactive P18 would also contribute to human MTC
tumorigenesis.

It has been suggested that p18 is a haploinsufficient gene in
mice. Haploinsufficiency of p18 has only been detected in the
presence of carcinogen treatment (20) or in combination with loss
of p27 or Patched (11, 34). In our mouse models, we show that p18
functions as a haploinsufficient tumor suppressor gene in the
presence of oncogenic RET. However, the haploinsufficiency of
p18 is not absolute because loss of both p18 alleles resulted in a
higher MTC incidence in all genotype groups and loss of p18Ink4c
expression resulted in enhanced MTC growth in p18−/− mice.
Taken together, we show that loss of p18 greatly contributes to
RET-induced MTC incidence as well as MTC progression. We
propose that loss of p18 (and p27) is an additional oncogenic hit in
the multistep process of RET-induced MTC tumorigenesis.

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References


Table 3. Loss of p18Ink4c expression in MTCs of RET2B;p18−/− (p27+/−) knockout mice correlates with plasma calcitonin levels and proliferation rates

<table>
<thead>
<tr>
<th>Genotype</th>
<th>p18Ink4c expression</th>
<th>Plasma calcitonin (pg/mL)</th>
<th>PCNA index* (total)</th>
</tr>
</thead>
<tbody>
<tr>
<td>RET2B;p18−/−</td>
<td>Positive</td>
<td>1.7</td>
<td>ND</td>
</tr>
<tr>
<td>RET2B;p18−/−;p27−/−</td>
<td>Positive</td>
<td>2.5</td>
<td>8.9% (603)</td>
</tr>
<tr>
<td>RET2B;p18−/−</td>
<td>Positive</td>
<td>2.6</td>
<td>10.3% (637)</td>
</tr>
<tr>
<td>RET2B;p18−/−;p27−/−</td>
<td>Positive</td>
<td>4.8</td>
<td>13.5% (635)</td>
</tr>
<tr>
<td>RET2B;p18−/−</td>
<td>Positive</td>
<td>12.7</td>
<td>9.6% (623)</td>
</tr>
<tr>
<td>RET2B;p18−/−;p27−/−</td>
<td>Positive</td>
<td>13.5</td>
<td>15.4% (633)</td>
</tr>
<tr>
<td>RET2B;p18−/−;p27−/−</td>
<td>Positive</td>
<td>19.5</td>
<td>11.0% (549)</td>
</tr>
<tr>
<td></td>
<td></td>
<td>Median, 4.8 pg/mL ‡</td>
<td>Mean, 11.5% (SD, 2.5)</td>
</tr>
<tr>
<td>RET2B;p18−/−;p27−/−</td>
<td>Negative</td>
<td>6.2</td>
<td>18.8% (645)</td>
</tr>
<tr>
<td>RET2B;p18−/−;p27−/−</td>
<td>Negative</td>
<td>13.0</td>
<td>14.6% (584)</td>
</tr>
<tr>
<td>RET2B;p18−/−;p27−/−</td>
<td>Negative</td>
<td>27.0</td>
<td>21.4% (496)</td>
</tr>
<tr>
<td>RET2B;p18−/−;p27−/−</td>
<td>Negative</td>
<td>29.0</td>
<td>10.5% (963)</td>
</tr>
<tr>
<td>RET2B;p18−/−;p27−/−</td>
<td>Negative</td>
<td>42.1</td>
<td>15.3% (625)</td>
</tr>
<tr>
<td>RET2B;p18−/−;p27−/−</td>
<td>Patchy</td>
<td>152.0</td>
<td>22.1% (569)</td>
</tr>
<tr>
<td>RET2B;p18−/−;p27−/−</td>
<td>Patchy</td>
<td>156.0</td>
<td>20.8% (2,495)</td>
</tr>
<tr>
<td>RET2B;p18−/−;p27−/−</td>
<td>Patchy</td>
<td>158.0</td>
<td>12.4% (593)</td>
</tr>
<tr>
<td>RET2B;p18−/−;p27−/−</td>
<td>Patchy</td>
<td>513.0</td>
<td>18.4% (645)</td>
</tr>
<tr>
<td>RET2B;p18−/−;p27−/−</td>
<td>Patchy</td>
<td>522.0</td>
<td>24.6% (530)</td>
</tr>
<tr>
<td></td>
<td></td>
<td>Median, 97 pg/mL ‡</td>
<td>Mean, 17.9% (SD, 4.6)</td>
</tr>
</tbody>
</table>

*PCNA index is an average from analysis of four microscopic fields per MTC.
† Total amount of cells analyzed.
‡ P = 0.001.
\ P = 0.007.
Synergistic Effect of Oncogenic RET and Loss of p18 on Medullary Thyroid Carcinoma Development

Wendy van Veelen, Carola J.R. van Gasteren, Dennis S. Acton, et al.


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